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Myogenic Potential of Murine Embryonic Stem Cells in the *Dmd*^{mdx} Mouse Model for Duchenne Muscular Dystrophy

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1. Introduction

The neuromuscular disorders are a heterogeneous group of genetic diseases, causing a progressive loss of the motor ability. More than 30 genetically defined forms are recognized, and in the last decade, mutations in several genes have been reported, resulting in the deficiency or loss of function of different important muscle proteins. Biochemical and immunohistological analysis have localized these proteins in several compartments of the muscle fiber. The proteins dystrophin, sarcoglycans and dysferlin are sarcolemmal or perisarcolemmal proteins, α 2-laminin and collagen VI are extracellular matrix proteins, telethonin and actin are sarcomeric proteins, calpain 3 and FKR^P are cytosolic enzymes, and emerin and lamin A/C are nuclear proteins (Vainzof et al., 2008).

Defects in components of the dystrophin-glycoprotein complex (DGC) are known to be an important cause of different forms of muscular dystrophies (Ervasti & Campbell, 1993; Yoshida & Ozawa, 1990). The DGC is an oligomeric complex which connects the subsarcolemmal cytoskeleton to the extracellular matrix. The DGC consists of dystroglycan (α - and β -DG), sarcoglycan (α , β -, γ -, δ - and ϵ -SG) and syntrophin/dystrobrevin subcomplexes. The intracellular link of the DGC is the protein dystrophin, that plays an important structural role in muscle fibers. Mutations in the dystrophin gene cause the most common form of X-linked Duchenne muscular dystrophy (DMD) (Hoffman et al., 1987). Dystrophin binds its amino-terminal and rod domain to actin and with its carboxy terminal to the integral membrane protein β -DG. The sarcoglycan sub-complex is also linked to β -DG and includes α -SG, β -SG, γ -SG, and δ -SG, which are tightly associated and inserted into the membrane. Mutations in the genes coding the 4 SG proteins cause severe forms of limb-girdle muscular dystrophies type LGMD2D, 2E, 2C and 2F, respectively. The peripheral membrane glycoprotein α -DG, a receptor for the heterotrimeric basement membrane protein laminin-2, binds to β -DG and so completes the connection from the inside to the outside of the cell (Straub & Campbell, 1997). Mutations in the LAMA2 gene, encoding the α 2 chain of laminin-2, cause α 2-laminin deficiency, and a severe form of congenital

muscular dystrophy (CMD1A) linked to chromosome 6q (Tomé et al., 1994). In addition, some forms of muscular dystrophy have recently been associated with genes encoding putative or known glycosyltransferases. Muscle protein analysis in these patients show a hypoglycosylation of α -dystroglycan and a consequent reduction of numerous ligands components of the extracellular matrix, such as laminin 2 (Muntoni et al., 2004). Other milder forms of muscular dystrophy are caused by mutations in genes coding the enzyme calpain 3 (LGMD2A), the sarcolemmal protein dysferlin (LGMD2B), and the sarcomeric protein telethonin (LGMD2G) (revision in Vainzof & Zatz, 2007).

Several animal models, manifesting phenotypes observed in neuromuscular diseases have been identified in nature or generated in laboratory. These models generally present physiological alterations observed in human patients, and can be used as important tools for genetic, clinic and histopathological studies (Vainzof et al., 2008)

The *Dmd^{mdx}* mouse is the most widely used animal model for Duchenne muscular dystrophy (DMD). Although it is a good genetic and biochemical model, presenting total deficiency of the protein dystrophin in the muscle, this mouse is not useful for clinical trials, because of its very mild phenotype. The canine golden retriever MD model represents a more clinically similar model of DMD due to its larger size and significant muscle weakness. Autosomal recessive limb-girdle MD forms models include the *SJL/J* mice that develop a spontaneous myopathy resulting from a mutation in the Dysferlin gene, being a model for LGMD2B. For the human sarcoglycanopathies (SG), the BIO14.6 hamster is the spontaneous animal model for δ -SG deficiency, while some canine models with deficiency of SG proteins have also been identified. More recently, using the homologous recombination technique in embryonic stem cell, several mouse models have been developed with null mutations in each one of the 4 SG genes. All sarcoglycan-null animals display a progressive muscular dystrophy of variable severity, and share the property of a significant secondary reduction in the expression of the other members of the sarcoglycan subcomplex, and other components of the Dystrophin-glycoprotein complex.

Mouse models for congenital MD include the *Lama2^{dy}/J* (dystrophin-muscularis) mouse, and the allelic mutant *Lama2^{dy2}/J* mouse, both presenting significant reduction of α 2-laminin in the muscle and a severe phenotype. The myodystrophy mouse (*Large^{myd}*), harbors a mutation in the glycosyltransferase Large, which leads to altered glycosylation of α -DG, and also a severe phenotype.

The study of animal models for genetic diseases, in spite of the existence of differences in some phenotypes can provide important clues to the understanding of the pathogenesis of these disorders and are also very valuable for testing strategies for therapeutic approaches.

In all forms of muscular dystrophies, in the early phase of the disease, there is a continuous process of degeneration and regeneration. The ability of adult skeletal muscle to regenerate has been attributed to the special properties of satellite cells. In mature skeletal muscle satellite cells are normally in a dormant state. Upon injury, many growth factors are secreted at the site of the lesion that recruits satellite cells, triggering the process of regeneration. In the progression of the dystrophic process, however, the endogenous satellite cell pool becomes exhausted and degenerated muscle fibers are replaced by fibrotic and adipose tissues, responsible for the observed muscle weakness.

Therefore, stem cell therapy should be an ideal treatment for all forms of muscular dystrophies, in which the deficiency of a specific muscle protein leads to muscle degeneration. Stem cell transplantation would either prevent or break the cycles of degeneration and regeneration in the dystrophic process, by replacing the deficient protein

(Meng et al., 2011). The majority of the therapeutic trial under investigation are done using Duchenne muscular dystrophy as a model for, because it is the most common and severe mendelian form of muscular dystrophy.

2. Stem cell therapy for muscular dystrophies

The use of normal stem cells to rescue the effects associated with mutant tissue is a promising avenue of research. To correct the dystrophic phenotype healthy stem cells transplanted to the diseased muscles must first be attracted to the injured area, answer to the endogenous signals that stimulate muscle differentiation and, ultimately, fuse to dystrophic myofibers, or form new myotubes. Incorporated healthy nuclei would thus contribute to restore the normal levels of functional skeletal muscle proteins such as dystrophin.

According to a recent revision by Meng et al. (2011), the ideal stem cell for treating DMD should fulfill several criteria, and should be able to: expandable in vivo, maintaining stem cell properties, be immune-competent, be systemically-delivered reaching all muscles of the body, survive, proliferate and migrate upon arrival within the host muscle, differentiate into muscle fibres, reconstitute the satellite cell pool with functional stem cells, be capable of expressing the missing protein, lead to improvement in muscle strength.

The most appropriated type of stem cell for these therapies is still under investigation. Among adult stem cells, skeletal muscle satellite cells have been considered the only source of stem cells for post-natal muscle regeneration (Seale & Rudnicki, 2000). Other stem cells within the skeletal muscle have, however been identified, including muscle-derived stem cells, muscle side population cells, myogenic endothelial cells, and mesoangioblasts (pericytes) (Meng et al, 2011). Human skeletal muscle-derived pericytes have been shown to form muscle fibers, after intra-arterial transplantation in the dystrophin deficiency host mice (Dellavalle et al., 2007). However, in contrast, muscle-derived cells (mdcs), which are very similar to the pericytes, did not contribute to muscle regeneration after systemic delivery in mdx nu/nu host (Meng et al., 2011b). These findings illustrate the opinion of several authors suggesting that further work is necessary to prepare pure cell populations from skeletal muscle that maintain their phenotype in culture and make a robust contribution to skeletal muscle regeneration after systemic delivery in dystrophic mouse models (Meng et al., 2011b)

Among the other non-muscle stem cells, previous attempts at developing stem cell therapies for the treatment of DMD were performed using bone marrow and blood-derived stem cells, mesenchymal stem cells from other origins, and pluripotent stem cells. Bone marrow-derived stem cells (BM) are a population of circulating cells with myogenic potential, present in the bone marrow, as already demonstrated as early as in the 1960s. Afterward, some authors confirmed that BM-derived cells can undergo myogenic differentiation and participate in muscle repair after injury, albeit at very low levels (Ferrari et al., 1998; Gussoni et al., 1999). Transplantation studies with BM cells injected into *Dmd^{mdx}* mouse confirmed that these cells may persist in the musculature for long periods of time, and that they are able to express exogenous dystrophin protein. However, the amount of muscle generated after a BM transplant was still not therapeutically relevant, consisting of about 0.5% of regenerating fibers containing donor cells (Ferrari et al., 2002; Gussoni et al., 2002). Therefore, none of these experiments provide consistent data with the idea that transplanted BM cells can actually correct the dystrophic phenotype.

Mesenchymal stem cells from other origins have been shown to regenerate muscle fibers, but at a very low efficiency, in mouse models. However, some therapeutic effects have been observed, such as by reducing inflammation in the dystrophin deficient muscle (Ichim et al., 2010).

Because of their pluripotency, much attention has been devoted to the potential applications of Embryonic Stem (ES) (Evans & Kaufman, 1981). However, to date, ES cells have not had a significant impact on the development of cell-based therapies to treat muscular dystrophy. While a few studies have been performed in *Dmd^{mdx}* mice, mainly involving the transplantation of embryoid bodies co-cultured with dissociated skeletal muscle, no evidence of long-term regenerative capacity of transplanted cells has been observed (Bhagavati & Xu, 2005). In table 1, the published experiments to date are summarized.

ES cell type	Strategy	Animal models	Markers	Delivery	Reference
mESC	media supplementation with DMSO	only in vitro experiments	none	only in vitro experiments	Rohwedel et al., 1994
mESC	IGF-II overexpression	only in vitro experiments	none	only in vitro experiments	Prelle et al., 2000
mESC	co-culture with muscle cells culture	<i>mdx</i> mice/ <i>Rag2</i> ^{-/-} <i>γc</i> ^{-/-} mice	none	intramuscular	Bhagavati & Xu, 2005
hESC	media supplementation 5-azacytidine	NOD-SCID mice	none	intramuscular	Zheng et al., 2006
ZHTc6 mESC	MyoD gene-inducible system	<i>mdx</i> nude mice	Sca ⁺ /c-kit ⁺ /CD34 ⁻	intramuscular	Ozasa et al., 2007
hESC	FACS of mesenchymal precursors	SCID/ <i>Beige</i> mice	CD73 ⁺ /CD56 ⁺	intramuscular	Barberi et al., 2007
mESC	Pax3 induction/ FACS	<i>mdx</i> mice/ <i>Rag2</i> ^{-/-} <i>γc</i> ^{-/-} mice	PDGFR ⁺ /Flk1 ⁺	intramuscular/systemic	Darabi et al., 2008
mESC	FACS of paraxial mesoderm precursors	<i>KSN</i> nude mice	PDGFR ⁺ /Flk1 ⁻ or Flk ⁺	intramuscular	Sakurai et al., 2008
mESC	media supplementation DMSO and RA	only in vitro experiments	none	only in vitro experiments	Kennedy et al., 2009
mESC	Pax3 induction/ FACS	<i>Frg1</i> mice	PDGFR ⁺ /Flk1 ⁺	intramuscular/systemic	Darabi et al., 2009
mESC	media composed of 5% HS and 10% FBS	<i>mdx</i> mice	SM/C-2.6 ⁺	intramuscular	Chang et al., 2009

Table 1. Summary of experiments for testing the myogenesis potential of ESCs in NMD. Thus, stem cell therapies for muscle disorders are still in the early days. There are many outstanding questions such as the immunogenic capacity of stem cells, whether local or systemic injections are the best route of administration in the diseased tissue, whether there is an optimal amount of cells to be administered, or whether the regenerative growth factors present in the dystrophic muscle are sufficient to promote the survival and the fusion of these cells to the damaged muscle (Bradley et al., 2002; Mimeault et al., 2007).

In order to answer at least some of the questions summarized above, we investigated the capacity of ES and EB cells to generate muscle in vivo, and to express the deficient protein dystrophin in the *Dmd^{mdx}* mouse model for DMD.

3. In vivo experiments with ES cells and potential therapies for NMD

3.1 ES cell culture

The embryonic stem cell line USP-1 derived from 129/Sv mice (Sukoyan et al., 2002) was cultivated as described (Rohwedel et al., 1994). Briefly, cells grew on irradiated mouse feeder layer on gelatin-coated flasks (Nunc) in DMEM (Gibco) supplemented with 10% fetal

bovine serum (Hyclone) and additives (Rohwedel et al., 1994). During the isolation and early stages of ES cell cultivation, the medium was supplemented with human recombinant leukemia inhibitory factor (hLIF) at 1000 units/ml (Chemicon) and was replaced every day. When reached the confluence (after 4 days in culture) these cells were trypsinized, and transferred to another culture flask (75 cm²) previously covered by a layer of mouse fibroblast feeders, plated at a density of 1.0×10^6 cells/cm² for proliferation. For differentiation, aliquots of 20 μ l cultivation medium containing 800 cells were placed on the lids of Petri dishes filled with PBS, based in protocols developed by Wobus et al. in 1988 (2002), which are based in previous differentiation by embryoid body formation and posterior treatment with 1% DMSO (dimethylsulfoxide). The ES cell aggregates (Embryoid Bodies - EB) were cultivated in hanging drops for 2 days and subsequently in suspension on bacteriological Petri dishes for additional 3 days in a specific skeletal muscle differentiation medium (proliferation medium with 1% DMSO, but no LIF). At day 5, EBs were plated separately onto gelatin-coated 24 well-microwell plates for morphological analysis. EB were dissociated before injection and ES cells were marked with red dye Vybrant® Dil cell-labeling solution (Invitrogen) to facilitate their tracking in injected muscles. This dye efficiently label live cells, and is diluted and disappears with cells proliferation and cellular death.

3.2 Animals, immunosuppression and transplantation

Four to six-week-old *Dmd^{mdx}* mice recipients were obtained from our animal house, in Human Genome Research Center at University of São Paulo. The mice received routine required cares for good health, and all experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo.

For the transplantation experiments, the *Dmd^{mdx}* mice were divided into groups according to cell treatment (Table 2). In the immunosuppressed group, the animals received a daily dose of 1 mg/kg FK506 (Tacrolimus, Sigma-Aldrich) intraperitoneal injections, from the day before stem cells injection until the time of euthanasia.

Each animal was injected with 1.0×10^6 ES or EB cells into the gastrocnemius muscle, or through intravenous injections in the tail vein.

Animals were euthanized using CO₂ chamber 2 days, 1 week, 2 weeks, 4 weeks and 8 weeks post-transplantation, and muscles were analyzed for dystrophin expression, and identification of the presence of the injected stem cells.

3.3 Tissue processing and dystrophin analysis

Gastrocnemius muscles were dissected and collected from all mice and additionally, several other tissues such as tail, liver and spleen were collected from the mice injected systemically. Samples of the contra lateral muscles were also collected and used as controls in the intramuscular injected mice. All tissue were immediately frozen in liquid nitrogen. For histological analysis, frozen sections of 7 μ m were prepared on slides using a cryostat (Zeiss, Jena, Germany).

Cryosections were stained with hematoxylin and eosin or used for immunohistochemistry to evaluate the expression of the muscle proteins. In brief, they were incubated with 1:100 diluted rabbit antibody anti-dystrophin AB 15277 (Abcam) and mouse antibody anti-fetal myosin NCL-MHCd (Novocastra). Cy3-conjugated anti-rabbit and anti-mouse (1:100) were used as secondary antibodies (Sigma-Aldrich). Samples were analyzed under fluorescence microscopy using appropriate filters for the fluorophore.

Treatment: ES cells		
	Administration via	Euthanasia and analysis
Nonimmunosuppressed mice		
Mdx 19	Local	2 days
Mdx 20	Local	2 week
Mdx 21	Local	4 weeks
Mdx 22	Systemic	2 weeks
Mdx 23	Systemic	4 weeks
Immunosuppressed mice		
Mdx 10	Local	1 week
Mdx 11	Local	2 weeks
Mdx 12	Local	4 weeks
Mdx 13	Systemic	1 week
Mdx 14	Systemic	2 weeks
Treatment: EB cells		
Immunosuppressed mice	Administration via	Euthanasia and analysis
Mdx 15	Local	4 weeks
Mdx 16	Local	8 weeks
Mdx 17	Systemic	4 weeks
Mdx 18	Systemic	8 weeks

Table 2. Intramuscular and systemic injected *Dmd^{mdx}* mice.

Total proteins were extracted from muscle and other tissues, separated by 6% SDS-PAGE polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membrane (GE Healthcare) for 60 min at 0.35A at 4°C. Membranes were then pre-stained in 0.2% Ponceau S, to ensure protein transfer and equal loading of the lanes with protein. Membranes were blocked with 5% nonfat milk in PBS, 0.1% Tween 20 (PBS-T) for 1 h and subsequently incubated with mouse antibody directed against dystrophin VP-D508 (Vector). After an overnight period of incubation with the primary antibody, membranes were washed three times with PBS-T for 10 min. The blots were then reacted with alkaline phosphatase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The detection of protein was done using colorimetric reaction for the enzyme, using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate (Vainzof et al., 1993).

Data on the injected cells (ES or EB cells), immunosupression and detection of cells both through red dye presence and DNA analysis, as well as screening for dystrophin expression in all injected animals are summarized in Table 3.

To check the ability of these cells to differentiate into new muscle fibers, and to express muscle proteins, we analyzed recipient muscles by immunohistochemistry using an anti-dystrophin antibody. The immunohistochemistry revealed no significant labeling, with only scattered fibers partially labeled, in a pattern observed also in non-injected *Dmd^{mdx}* mice (Fig. 1). Western blot analysis confirmed the lack of dystrophin expression in all tested animals.

Local injections										
ES						EB cells				
Analysis			Detection			Analysis		Detection		
	time	red dye	DNA	Dystrophin		time	red dye	DNA	Dystrophin	
Nonimmunosuppressed mice	Mdx 19	2 d	+++	-	-					
	Mdx 20	2 w	-	-	-					
	Mdx 21	4 w	-	-	-					
Immunosuppressed mice	Mdx 10	1 w	+++	-	-	Mdx 15	4 w	-	-	-
	Mdx 11	2 w	++	+	-	Mdx 16	8 w	-	-	-
	Mdx 12	4 w	+	-	-					
Systemic injections										
ES						EB cells				
Analysis			Detection			Analysis		Detection		
	time	red dye	DNA	Dystrophin		time	red dye	DNA	Dystrophin	
Nonimmunosuppressed mice	Mdx 22	2 w	-	-	-					
	Mdx 23	4 w	-	-	-					
Immunosuppressed mice	Mdx 13	1 w	+	(tail)	-	Mdx 17	4 w	-	-	-
	Mdx 14	2 w	+	(tail)	-	Mdx 18	8 w	-	-	-

Table 3. Results observed in the injected mice.

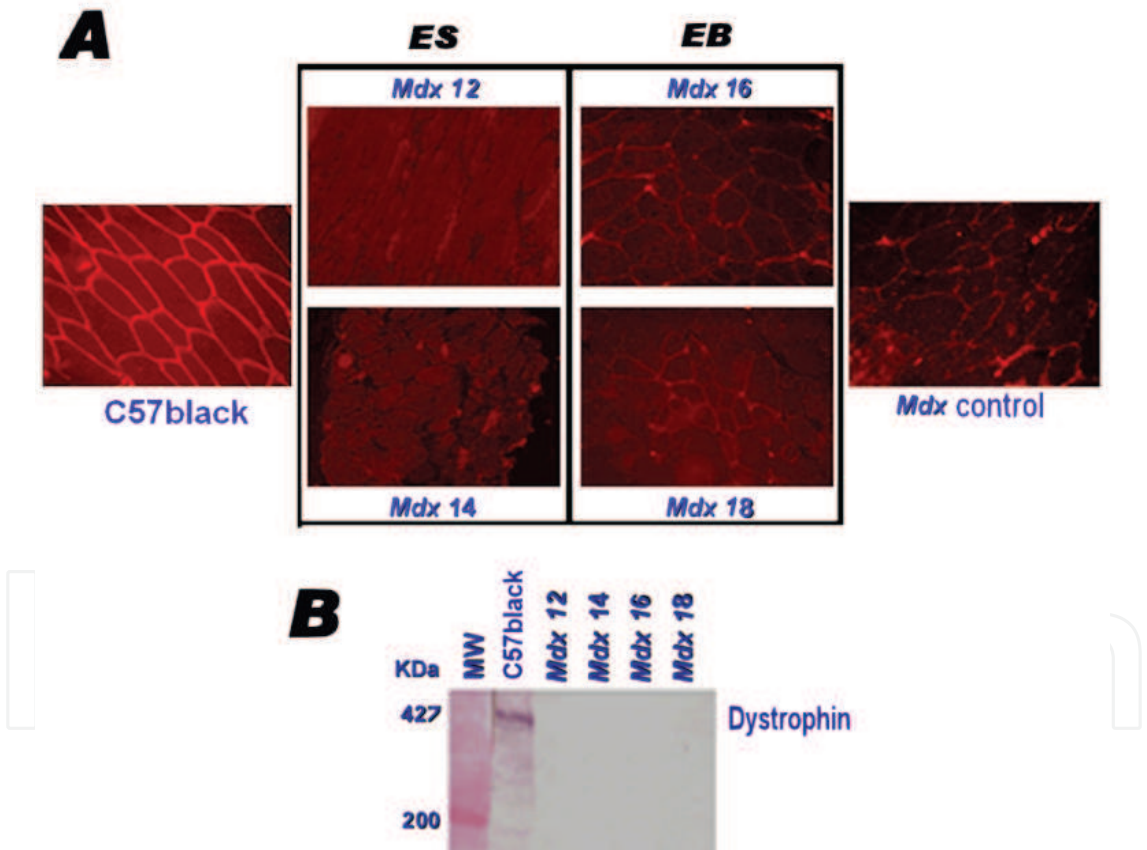


Fig. 1. Dystrophin analyses in the injected and control muscles. (A) Dystrophin immunofluorescence and (B) Western blotting analysis showing the positive pattern of labeling in normal muscle of *C57black* mice and the absence dystrophin in immunosuppressed mice injected with ES (Mdx12 and Mdx14) and EB cells (Mdx 16 and Mdx 18). Cells were injected in the muscle in Mdx 12 and Mdx 16, and intra-venous in Mdx 14 and Mdx 18.

3.4 ES and EB cells identification

Genomic DNA was obtained from cells and muscle tissue of injected mice. Fragments of injected and not injected muscles were placed in an extraction solution (100mM Tris HCl pH 8.5; 5mM EDTA, 0.2% SDS; 200mM NaCl), containing 2.5 μ l proteinase K (10mg/ml). These samples were kept overnight in dry bath at 55°C. The samples were subjected to centrifugation of 1000g for 10 minutes, and added 500 μ l isopropanol in the supernatant. After the new centrifugation of 13500g for 15 minutes, the DNA was precipitated in TE buffer (TRIS-EDTA). The PCR primers used were from the same region of the genome of mice, which produce products of different sizes due to the presence of polymorphic regions, referring to strains of 129/*Sv* mice and *Dmd*^{mdx} mice [<http://www.informatics.jax.org/searches/probe.cgi?37495>] for the identification of ES cells. PCR conditions were 95°C 5 minutes, with 40 cycles of 95°C 1 minute, 55°C 1 minute and 72°C 1 minute. DNA fragments were separated by electrophoresis in 10% acrylamide gels and marked with ethidium bromide to its visualization under UV transillumination.

As almost 100% of the injected ES and EB cells were previously labeled with a red fluorescent dye, we analyzed the presence of these marked cells in histological sections of the injected mice. We identified their presence for longer periods (after 1 week) only in the muscle of the ES injected mice (Table 3 and Fig. 2). This may mean that the ES remained in the injected place.

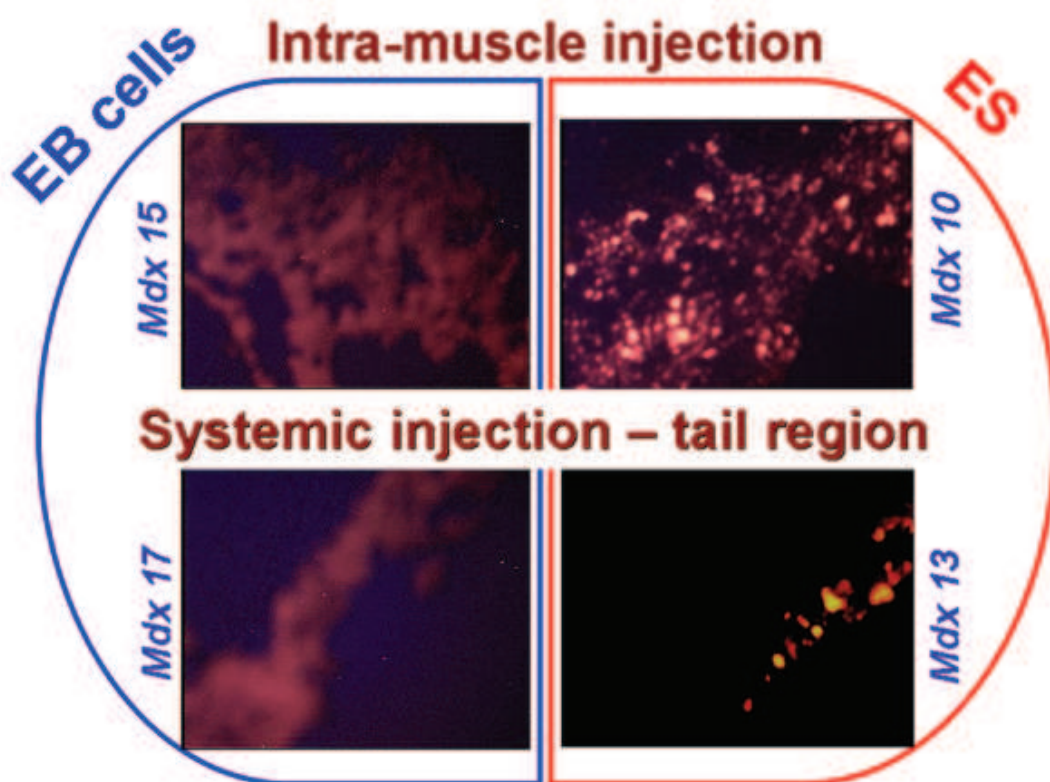


Fig. 2. Identification of dye labeling of ES and EB cells. Screening for the red-labeled ES and EB cells injected in the different *Dmd*^{mdx} mice. Intramuscular injections (Mdx 15 and Mdx 10) and systemically-injected (Mdx 17 and Mdx 13), showing that only the ES cells were identified.

In the animals Mdx 10, 11 and 12 (local injections of ES cells, after 1, 2 and 4 weeks), we noticed an increase of the muscles in the leg, and the hematoxylin-eosin (H&E) staining showed an intense degeneration and presence of several types of undifferentiated cells (Fig. 3). A histopathological examination of frozen sections identified the proliferation of various types of immature cells, suggesting the formation of a tumor, consistent with the embryonic nature of these cells. But we did not have clear evidences that it was teratoma.

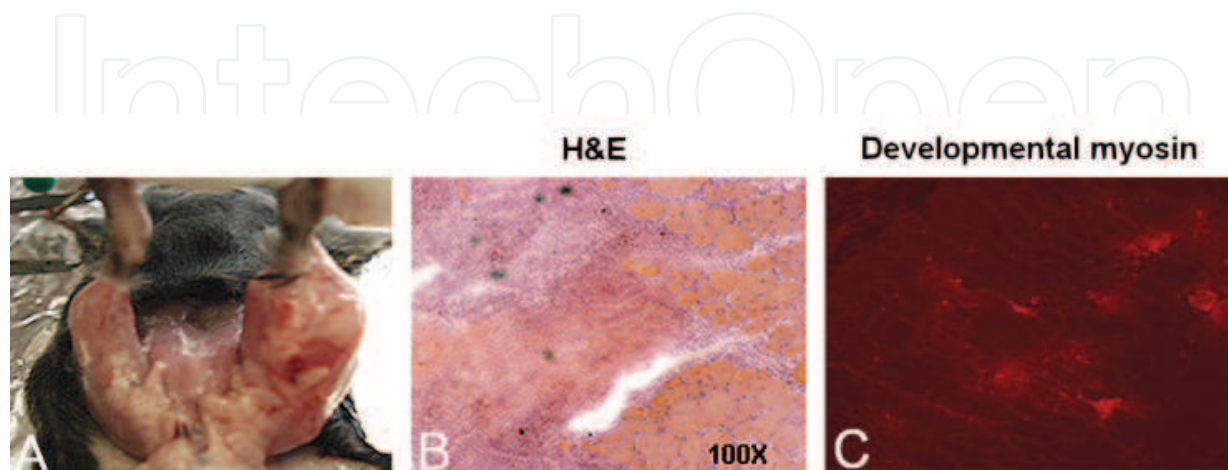


Fig. 3. Macroscopic and microscopic analyses of the muscle injected with ES cells. Analyses of the immunosuppressed Mdx 11, with local injections of ES cells, after 2 weeks post-transplantation. A- A significant increase in the muscles of the injected leg was observed. B- Hematoxylin-eosin (H&E) staining showed the presence of several types of undifferentiated cells. C- Immunohistological reaction using anti-developmental myosin showed no positive evidence of the presence of new fibers.

To check if the new generated tissue included differentiation into muscle fibers, we probed the same region described above with the antibodies against fetal myosin. However, there was no immunoreactivity for fetal myosin and no evidence of formation of new fibers (Fig. 3).

3.4.1 ES cell quantification

To investigate the presence and try to quantity of injected ES and EB cells in the muscle we used PCR analysis with specific primers to polymorphic markers of each cell strain. This method detected the presence of stem cells only in the muscle of Mdx 11 (Fig. 4A), who also showed an enlargement of the leg, with significant mass growth.

The experiment revealed a 121 bp band corresponding to the molecular weight of *Dmd^{mdx}* mice polymorphism, while a band of 142 bp is observed in the donor ES cells strain (line 4, Fig. 4A).

To estimate the relative amount of the injected cells and verify their possible multiplication in vivo, we performed a standard curve with known concentrations of DNA from the two different mice strains (Fig. 4B). The ES cells strain was only detected starting with the concentration of 30%. In Mdx 11, the number of ES cells in the mice injected muscle were in a similar concentration of the limit of detection of 30% (Fig. 4A). No 142 bp band was observed in other mice, suggesting lower concentration of the injected ES and EB cells in these muscles.

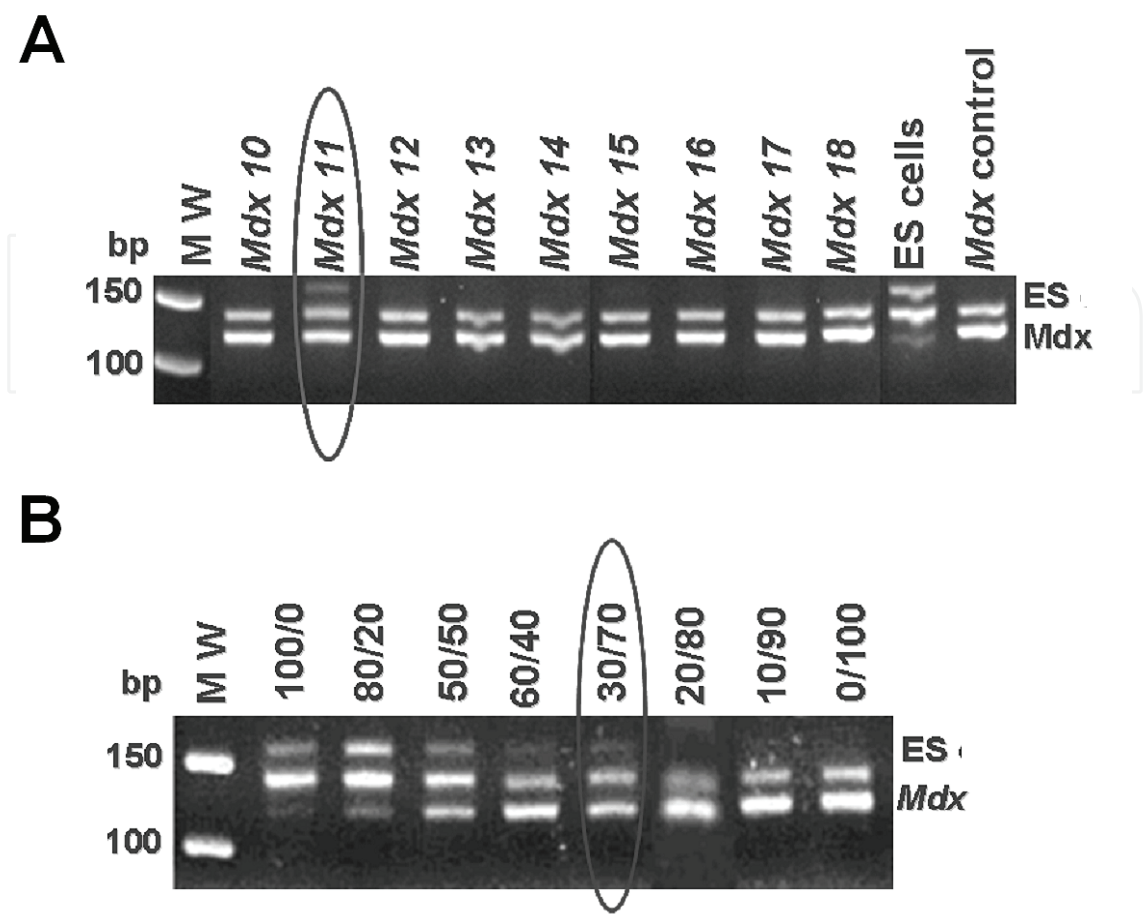


Fig. 4. DNA screening for the ES and EB presence. (A) PCR reaction for polymorphic markers of *C57Black* (*Dmd^{mdx}* strain) and *129/Sv* (ES and EB cells) strains in the muscles from all injected animals (all immunosuppressed). The band of 121 bp is from the *Dmd^{mdx}* strain, while the band of 142 bp is from of the *129/Sv* strain of the ES cells. In Mdx 11 both bands can be observed, indicating the presence of the ES cells in the injected muscles. (B) The same PCR reaction, in a standard curve with known concentrations of DNA from the two different mice strains, showing the identification of the ES cells starting in the concentration of 30%.

4. Efficient stem cell therapies for dystrophic muscle

Replacement therapies using stem cells are emerging as promising avenues for treatment of genetic diseases because the transplantation of healthy stem cells into affected individuals can potentially rescue defects and injuries.

The identification of mesenchymal stem cells (MSCs) as a sub-population of fibroblast-like adherent cells in the BM opened new perspectives for therapies for the different forma of muscular dystrophies, since these cells were able to differentiate into several other lineages, producing in vitro skeletal muscle, in addition to osteoblasts, chondroblasts, and adipocytes (Friedenstein et al., 1966; Prockop, 1997). Very recently, we showed that hMSC were not rejected when transferred to *SJL/J* mice by systemic and repeated injections (Vieira et al., 2008), while mMSC intramuscularly injected cells were eliminated after 2 days in the *Dmd^{mdx}* mouse (Ayub-Guerrieri et al., 2009). The injected cells in the *SJL/J* mouse were able to fuse

with the host muscle cell and to express the exogenous protein. However, in this previous study, the number of cells and injections were significantly higher and the *SJL/J* mouse model for LGMD2B presents a milder phenotype with no active muscle degeneration. This suggests that either the number of injected cells must be higher, or dystrophin deficient dystrophic muscle probably constitutes an adverse environment for the newly introduced cells. Thus, the high degree of degeneration, sclerosis and fat infiltration, as well as inflammation and activation of cascades of degeneration/regeneration genes of advanced muscular dystrophy reduce the efficacy of cell delivery, and can result in their poor survival in the injected muscles and limit the effectiveness of replacement therapies with stem cells. A very recent study (Gargioli et al., 2008) corroborates this hypothesis. Modified tendon fibroblasts expressing angiogenic factors and metalloproteinases were injected in *Dmd^{mdx}* mice and were able to restore the vascular network and reduce collagen deposition, allowing efficient cell therapy in aged dystrophic mice (Gargioli et al., 2008). Therefore, we suggest that efficient stem cell therapies for degenerated dystrophic muscle must include additional procedures to improve the general health of the diseased tissue before stem cells can be delivered and integrated.

Here we show that undifferentiated ES cells hold a better promise of success of retention than differentiated EB cells, since after intramuscular injections, ES cells, but not EB cells, were found in the muscle of animals after 1, 2 and 4 weeks post-injections. Additionally, systemic injection of both ES and EB cells were not directed nor retained by the dystrophic muscles. Two other reports on the use of ES cells in the treatment of DMD showed evidence for engraftment (Bhagavati & Xu, 2005; Darabi et al., 2008). In the first study, the transplantation of EB co-cultured with dissociated skeletal muscle into mdx mice was limited to qualitative detection of donor-derived cells in recipient muscle. However, in the second study, authors showed that paraxial mesoderm is not generated efficiently during ES differentiation induced by conventional protocols, which may further explain our lack of success in integrating ES cells into dystrophic muscles (Darabi et al., 2008).

A second important question relates to the extent to which stem cells are immunogenic, since the safe and successful clinical will be indirectly dependent on the strength of rejection reactions (Mimeault et al., 2007; Trounson 2006). The first studies on the immunologic properties of ES cells and their derivatives have suggested their ability to induce immunologic tolerance (Drukker et al., 2002; Bonde & Zavazava, 2006). Because of this, we investigated the myogenic potential of ES and EB cells when injected into muscle of immunosuppressed mdx mice. Our results showed that the non differentiated ES cells were retained, while pre-differentiated EB cells were all eliminated. Although this could happen because of the non-integration of the EB cells, this could also suggest that even with immunosuppression, more differentiated cells may cause a stronger immunological response than undifferentiated ES cells, as also suggested by other group (Drukker et al., 2002). These data are in accordance with recent publication showing that hESCs were highly immunogenic, triggered both cellular and humoral-mediated pathways, and as a result, were rapidly rejected in xenogeneic hosts. These authors observed that combinations of immunosuppressive regimens are necessary for stem cell survival in vivo (Swijnenburg et al., 2008).

Several reports have already described that, when injected sub-cutaneously into severe combined immunodeficient mice, undifferentiated ES cells will produce teratoma or teratocarcinoma, with derivatives of the three germ layers (Bradley et al., 2002; Mimeault et al., 2007; Reubinoff et al., 2000; Andrews et al., 2005; Wu et al., 2007). Here we observed an

increase of the leg of ES cells injected mice. Our histology findings suggest that the donor cells may have multiplied and have been embedded in the mice muscles, in a pattern similar to a teratoma. In one hand, the formation of tumor confirms the ability of these ES injected cells to multiplication in vivo. On the other hand, this new tissue had no muscle characteristics, since no dystrophin, nor developmental myosin positive fibers were identified. Ultimately, it is clear that the signaling environment of the severely dystrophic muscle was not able to induce the differentiation of stem cells into muscle cells.

The use of single injections of stem cells for future therapies in children would have the significant benefit of being less invasive, and less traumatic from a clinical and immunological stand point. But, it also leads to a limitation in the number of injected cells, which can be insufficient to promote an appropriate restoration of the degenerated muscle. However, the finding of the injected ES cells in the recipient muscle, even using only one application of the same quantity of 10^6 cells, indicates that this number was sufficient for their retention in the injected muscle. Using polymorphic markers for the ES cell line we identified a concentration of at least 30% of these cells in the injected muscle. This result indicates that muscle enlargement observed in injected mice was caused by at least this amount of cells. In the other locally injected mice with leg enlargement, probably a lower amount of these cells were sufficient to promote the tumor, but this amount was not sufficient to allow its detection by our methodology.

5. Conclusion

Our results suggest that the dystrophic muscle is an adverse ambient for the homing and maintenance of injected stem cells, since when the “still pluripotent” stem cells were injected they formed tumors, and when the muscle pre-differentiated cells were injected they were cleared/migrated out of the tissue. Injected embryonic stem cells, therefore, must receive more or different stimulation from the dystrophic environment to differentiate or to fuse into muscle. Additional studies are necessary to increase the therapeutic potential of these cells in dystrophic murine models.

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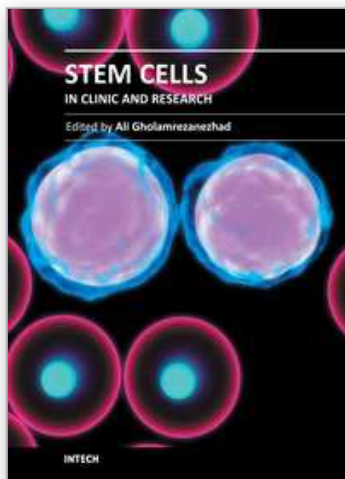
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Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigational more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

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